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# ENZYME PRODUCTION ON CELLULOSE/XYLOSE MIXTURE

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## ABSTRACT

Cellulase production by the RUT C-30 mutant of the fungus Trichoderma reesei was studied on mixtures of xylose and cellulose, both in batch and fed-batch system. In mixed substrates, the lag phase of the growth cycle was shorter, and reached the maximum of total productivity in a shorter time, compared to growth on the single substrate, cellulose. A diauxic pattern of utilization of the two carbon sources was observed as well: xylose was utilized first to support growth, followed by cellulose to induce the cellulase enzyme production and provide an additional carbon source for cellular metabolism. Of the various mixtures of xylose and cellulose used in batch enzyme production, a ratio of 30:30 g/l of xylose to cellulose was optimal. This mixture produced the highest maximal enzyme productivity of 122 IFPU/l/hr, and its total productivity reached a maximum value of 55 IFPU/l/hr in less time than others. However, similar total productivities and higher enzyme titers were observed for growth on cellulose alone. In the fed-batch system, a start up mixture of 30:20 g/l xylose to cellulose and an intermittent feeding mixture of 5:15 g/l/day xylose to cellulose (total of 20 g/l/day) produced the highest titer of enzyme activity of 12.5 IFPU/ml, and total productivity of 45.4 IFPU/l/hr.

## ENZYME PRODUCTION ON CELLULOSE/XYLOSE MIXTURE

### INTRODUCTION

Enzymatic hydrolysis is of interest for fuel production because it avoids many of the problems experienced with dilute acid hydrolysis, a method which has received attention since early 1900's. A key cost element in this process is the production of hydrolytic enzymes, and advances are required that will allow production of high cellulase titers at high rates with lignocellulosic feedstock as the carbon source. Detailed studies of current cellulose technology (Wright et al 1986) shows that approximately 25% of the enzyme production cost is for production of glucose from cellulosic materials for feeding the cellulase producing microorganisms.

A major improvement in cellulase technology was the development of the RUT C-30 and RL-P37 mutants of Trichoderma reesei (Montenecourt and Eveleigh 1977), which yield high enzyme activity and productivity. Currently, cellulase enzymes are typically produced by growing up the various mutants of the fungus T. reesei on cellulose (Ryu and Mandels 1980, Ghosh et al 1982). However, since cellulose is not easily assimilated, the growth rate of the fungus is slow, and batch times on the order of 1-2 weeks are required to achieve high enzyme titers. On the other hand, liquid carbon sources result in faster growth of the microorganisms, but T. reesei does not produce suitable enzyme activity when grown on inexpensive noninducing soluble sugar streams that are readily available in large quantities such as xylose (Allen and Mortensen 1981).

If the fungus could be grown to suitable cell densities on an inexpensive carbon source that is abundant and fed cellulosic substrate to induce cellulase production, perhaps both high growth rates and high enzyme titers could be achieved. The large amounts of xylose produced from biomass during pretreatment could be used to promote rapid cell growth and the resulting dense culture could then be fed cellulose to induce cellulase production. In this fashion, an inexpensive liquid carbon source will support rapid cell growth while the addition of cellulose could result in production of high enzymatic activity.

To determine the potential of this enzyme production scheme, the RUT C-30 mutant of the T. reesei was grown on mixtures of xylose and cellulose both in batch and fed-batch cultures. Even though more advanced mutants of T. reesei have been developed, RUT C-30 was selected for this study since considerable information is available on its growth and enzyme production. The objective of the research is to delineate the optimal concentrations and ratios of these substrates for the induction of the cellulase enzyme complex produced in batch and fed-batch cultures containing different initial concentrations of pure xylose and pure cellulose and to compare the results to those possible with cellulose alone.

### MATERIALS AND METHODS

#### Production Medium

Three types of mineral media (medium A, B, and C) were used in these

experiments as shown in Table 1. These media were the same as that of Tangnu et al (1981) and Wiley (1985) except peptone was replaced with corn steep liquor (Sigma Chem. Co., St. Louis, Mo.) as recommended by Sheir-Neiss and Montenecourt (1984). Medium A was used for starting fungus from frozen stock culture, medium B was used for preinocculum preparation, and medium C was used for main inoculum and production medium for fermenter. The pH of all the culture media was adjusted to 4.8 before autoclaving. Xylose (Sigma Chemical Company, St. Louis, Mo) and solka floc BM200 (James River Corporation, Berlin, New Hampshire) were used as carbon source.

Fermentation

Fermentations were carried out in a 5-L fermenter (B. Braun, Biostat V) with an operating volume of 2.5 L for batch culture and 2L for fed-batch culture using medium C of Table 1. Temperature was held constant at 28°C, and pH was controlled at 4.8 by addition of $NH_4OH$ and $H_3PO_4$ stocks, respectively. Dissolved oxygen was automatically controlled above 20% of the saturation value for the medium by varying the agitation rate or supplying pure oxygen instead of air. The foaming was controlled by addition of (1:20) solution of Antifoam B emulsion (Sigma Chemical Company) whenever it was needed. To minimize contamination by bacteria, 2 ml of antibiotics (penicillin and streptomycin, 5 mg/ml) were added per liter of fermenter volume after the fermenter and its contents were sterilized.

In fed-batch experiments, the fermentation was initiated as a conventional batch using mixture ratio of 30:20 g/l xylose to cellulose. After 48 to 72 hours, when the growth was observed to slow down, as demonstrated by a decrease in base addition, specified amounts (10 to 30 g/l, as shown in Table 2) of mixture of xylose and cellulose or cellulose alone were added to the fermenter. This intermittent addition was repeated on a daily basis. The total effective cellulose concentration was raised to as high as 250 g/l by this approach.

## ANALYSIS

Dry Weights

Five ml of culture broth was centrifuged, washed with distilled water, then dried in an aluminum dish overnight at 90°C. From the difference of weights, the total dry weight, which included mycelium and residual cellulose, was then determined. Mycelium dry weight was estimated indirectly from the protein content of the mycelium, using a correlation factor of 0.37 (protein g/l/dry cell weight g/l) which was determined in this work and described in the results section. Free cellulose was determined from the difference of total dry weight and mycelium dry weight.

Filter Paper Activity

Filter paper activity, expressed as international units (IFPU), was measured by the method recommended for the International Union of Pure and Applied Chemistry (1984).

Table 1: Growth Media Composition

| Component | Medium | A | B | C |
|---|---|---|---|---|
| Glucose | | 1.0% | - | - |
| Cellulose | | - | 1.0% | 5.0% |
| $CaCl_2.2H_2O$ | | 0.4 g/l | 0.4 g/l | 0.8 g/l |
| $MgSO_4.7H_2O$ | | 0.3 g/l | 0.3 g/l | 0.6 g/l |
| $KH_2PO_4$ | | 2.0 g/l | 2.0 g/l | 3.7 g/l |
| $(NH_4)_2SO_4$ | | 1.4 g/l | 1.4 g/l | 11.7 g/l |
| Corn Steep Liquor | | 1.5% | 1.5% | 1.5% |
| Tween 80 | | - | 0.2 ml/l | 0.2 ml/l |

Trace Mineral Concentrations

| | |
|---|---|
| $FeSO_4.7H_2O$ | 5. mg/l |
| $MnSO_4.H_2O$ | 1.6 mg/l |
| $ZnSO_4.7H_2O$ | 1.4 mg/l |
| $CoCl_2.6H_2O$ | 3.7 mg/l |

Prepared as Stock Solution of 100X Concentration and Used 10ml per liter

Antibiotics

Penicillin

Streptomycin

Prepared 5 mg/ml stock solution and used 2ml/l

## Soluble Protein

Cellular protein was measured by modified Lowry method (Markwell et al 1981). Bovine serum albumin was used as a standard.

# RESULTS

In this work, the T. reesei mutant, RUT C-30, was grown in batch and fed-batch fermentations on a mixture of xylose and cellulose, using a 10% v/v, 72 hours vegetative inoculum. The age of the inoculum, the amount of antifoam added to fermenter, and the rate of stirring affected enzyme production, as reported by other researchers (McLean and Podrazny 1985). Different ratios of xylose to cellulose, shown in Table 2, were used as the substrate. As a control, several experiments were performed in which only xylose or cellulose were used as substrates.

## Batch Cultivation

Figure 1 is the example of growth patterns of RUT C-30 on a mixture of xylose and cellulose (30:30 g/l). Figure 2 summarizes the cellulase activity in IFPU/ml as a function of time for all batch culture experiments. High titer filter paper activity, which is the maximum of enzyme activity, and the correspondent productivity along with maximal productivity were estimated from this figure, as shown in Table 3. Maximal productivity was calculated from the slope of the steepest part of Figure 2. Total productivity is based on the total time of fermentation including the lag phase, and for this reason, its values are lower than maximal productivity.

Study of the results shown in Table 3 indicates that the highest enzyme activities for all batch culture experiments with mixed substrates and a total carbon source of 50 g/l or more were similar (5-7 IFPU/ml). The experiment with a mixture ratio of 30:30 g/l xylose to cellulose produced the highest maximal productivity (122.7 IFPU/l/hr) for a titer of 5.6 IFPU/ml in 6.25 days. The maximal productivity obtained in experiments with substrate ratios of 25:25 and 30:20 g/l are lower than those of experiments with 30:30 and 20:20 g/l of xylose and cellulose respectively (Table 3). This difference may be explained by uncertainty involved in measurement of the slope, an inaccurate approximation itself.

Figure 3 shows the change of total productivity as a function of time for all batch culture experiments. This figure indicated that the total productivity for mixed substrates reached its maximum in less time than the time required for the single substrate, cellulose, although the final values are similar. The reason for this phenomena is that in mixed substrate experiments, xylose supports the growth and cellulose induces the enzyme production, making the overall process faster. However, for the single substrate, cellulose, the inducible substrate is required to both support growth and induce enzyme production, and since cellulose is broken down slowly, more time is required for growth. On the other hand, higher activities are obtained for growth on cellulose alone than for the mixed substrate, and since this compensates for the faster growth of the fungi, total productivities for cellulose alone or mixed substrate are similar.

Table 2: Substrates Used in Enzyme Production Experiments

| Batch Cultivation | Start-Up Concentration | | Intermittent Feeding Concentration | |
|---|---|---|---|---|
| Experiment # | Xylose (g/l) | Cellulose (g/l) | Xylose (g/l day) | Cellulose (g/l day) |
| 1 | 40 | 0 | | |
| 2 | 30 | 5 | | |
| 3 | 30 | 10 | | |
| 4 | 20 | 20 | | |
| 5 | 25 | 25 | | |
| 6 | 30 | 20 | | |
| 7 | 30 | 30 | | |
| 8 | 50 | 50 | | |
| 9 | 0 | 40 | | |
| 10 | 0 | 50 | | |
| 11 | 0 | 100 | | |
| **Fed Batch Cultivation** | | | | |
| 1 | 30 | 20 | 0 | 10 |
| 2 | 30 | 20 | 0 | 20 |
| 3. | 30 | 20 | 0 | 30 |
| 4. | 30 | 20 | 5 | 15 |
| 5. | 30 | 20 | 10 | 10 |
| 6. | 30 | 20 | 15 | 5 |
| 7. | 30 | 0 | 0 | 20 |
| 8. | 30 | 30 | 0 | 30 |

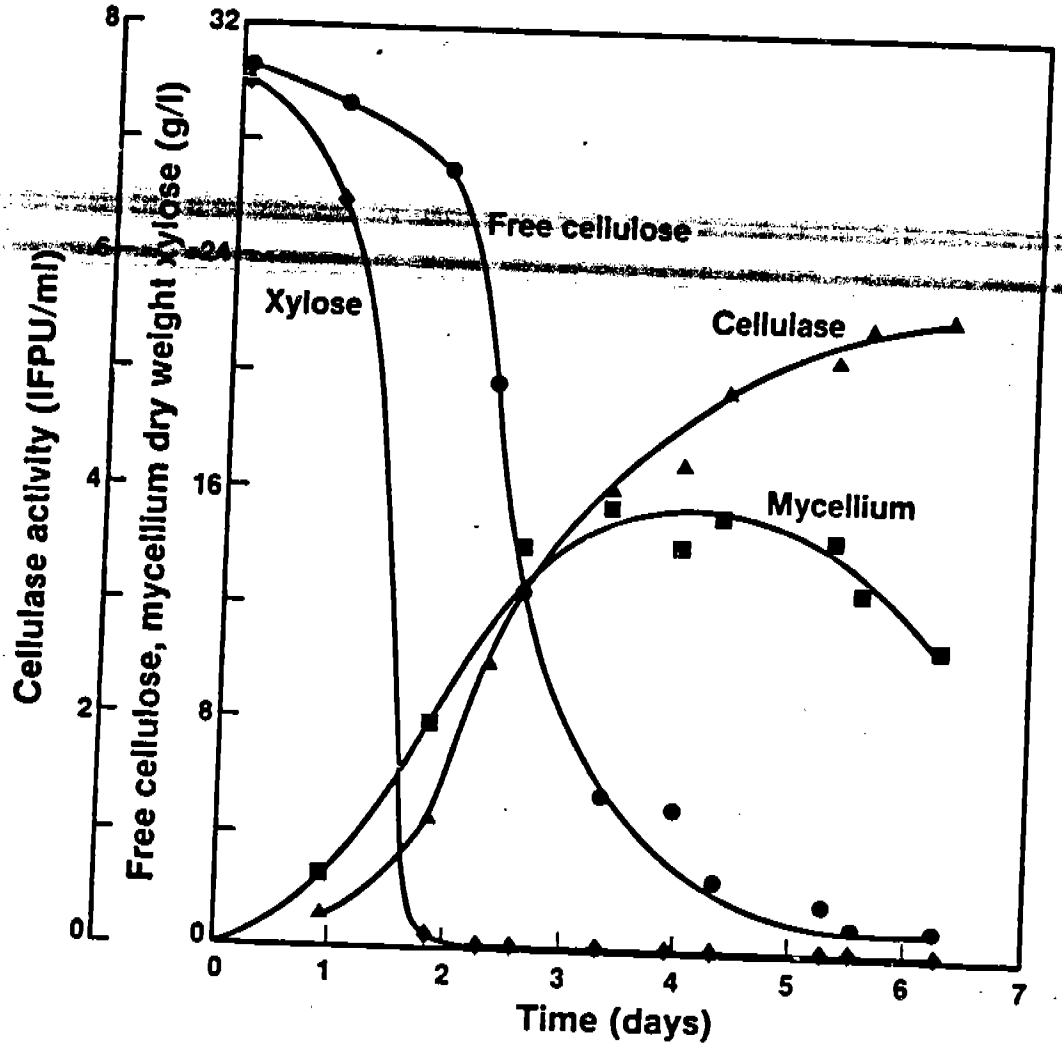



Figure 1: Growth and Enzyme Production Pattern of T. reesei RUT-C30 Grown on Mixture of Xylose:Cellulose (30:30 g/l) at pH = 4.8, T = 28°C.

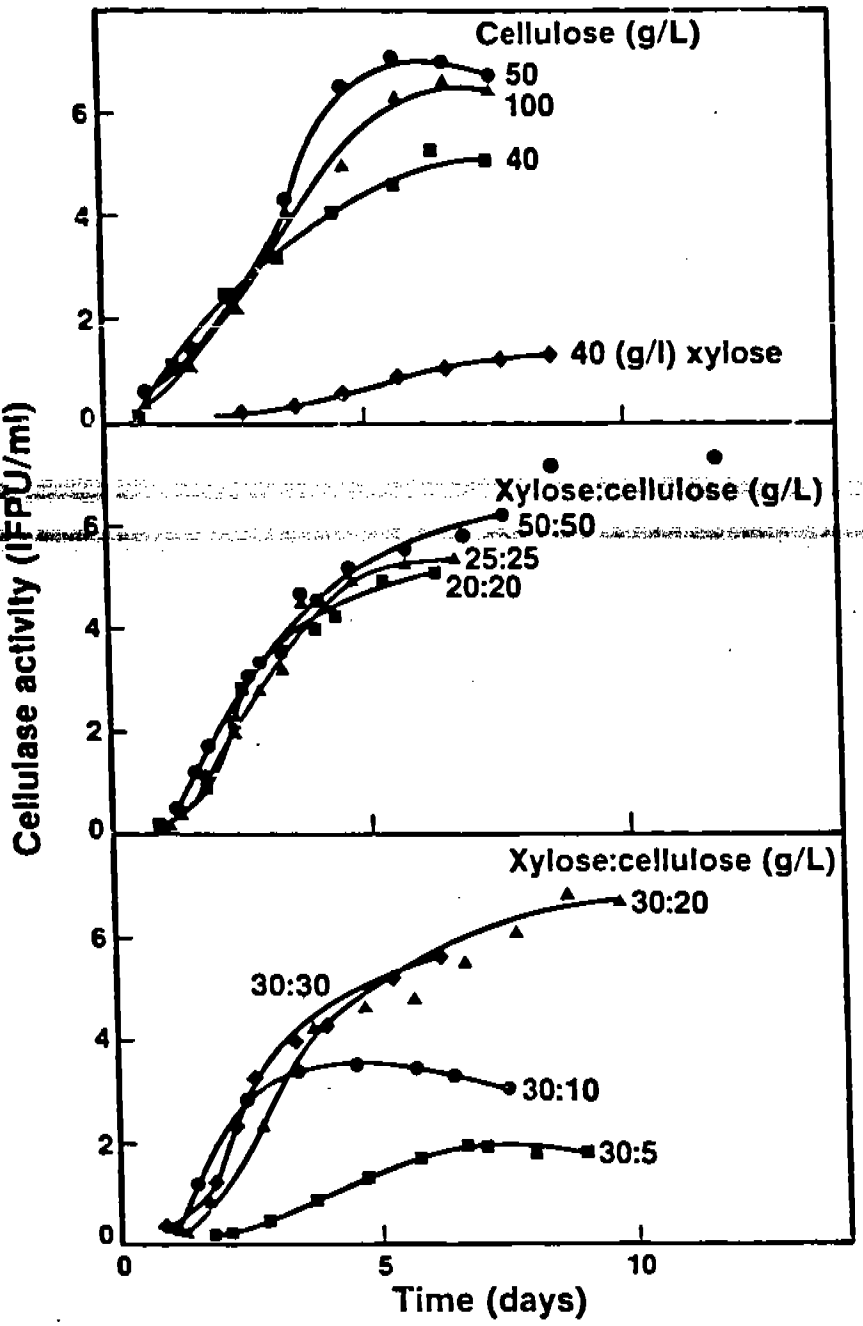



Figure 2: Cellulase Production as a Function of Time by T. reesei RUT-C30 Grown on Different Substrate in Batch Culture at pH = 4.8, T = 28°C.

Table 3: Summary of Results on the High Titer Filter Paper Activity, Correspondent Productivity, and Maximal Productivity of RUT-C30 Grown in batch mode on Xylose, Cellulose, or Their Mixture

| Exp.# | Xylose: Cellulose Ratio (g/l) | High Titer Activity (IFPU/ml) | Productivity (IFPU/l. hr) | |
|---|---|---|---|---|
| | | | Total | Maximal |
| 1 | 40:0 | 1.3 | 6.2 | 12.5 |
| 2 | 30:5 | 1.95 | 12.1 | 18.5 |
| 3 | 30:10 | 3.5 | 32.0 | 70.8 |
| 4 | 20:20 | 5.2 | 39.2 | 111.0 |
| 5 | 25:25 | 5.4 | 35.0 | 71.0 |
| 6 | 30:20 | 6.9 | 37.2 | 77.1 |
| 7 | 30:30 | 5.6 | 37.3 | 122.7 |
| 8 | 50:50 | 7.4 | 35.2 | 79.2 |
| 9 | 0:40 | 5.3 | 34.3 | 54.2 |
| 10 | 0:50 | 7.1 | 51.0 | 91.7 |
| 11 | 0:100 | 6.7 | 41.4 | 66.7 |

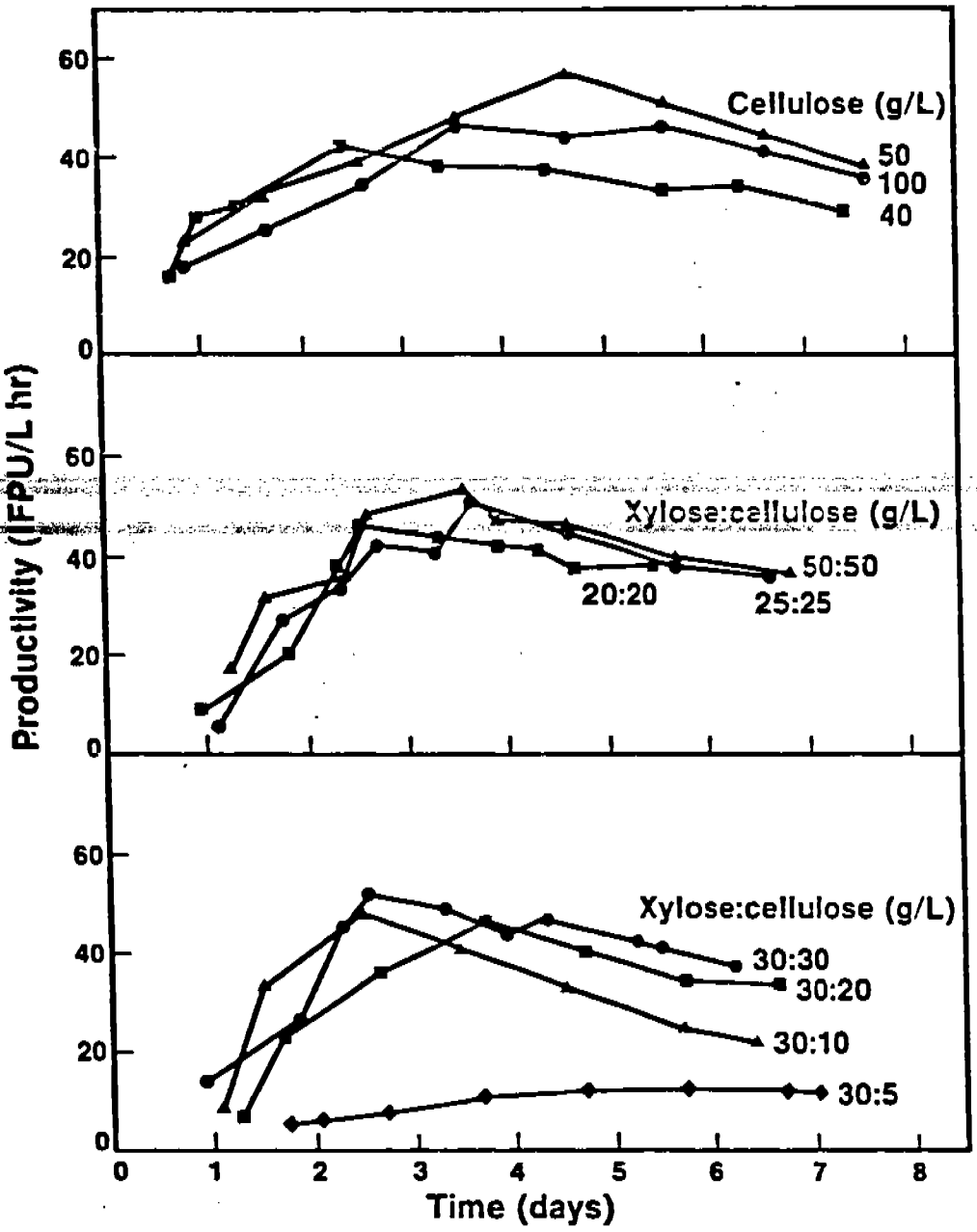



Figure 3: Productivity of Cellulose Production as a Function of Time for T. reesei RUT-C30 Grown on Different Substrate in Batch Culture at pH = 4.8, T = 28°C.

Our results also show that total productivity for mixed substrates reaches its maximum before enzyme activity gets to its high titer value, Figure 4. In this figure, as an example, the total productivity and enzyme activity are plotted versus time for experiments with 30:30 g/l xylose:cellulose, and 50 g/l cellulose.

Comparing the results of three experiments in which RUT C-30 was grown on xylose (40 g/l), cellulose (40 g/l), and a mixture of the two (20:20 g/l xylose:cellulose) (Table 3) shows that by substituting 20 g/l xylose for 20 g/l of cellulose, the maximal productivity has improved by 100%. Also, the other results (Table 3) show that 30:20 and 30:30 g/l mixtures of xylose and cellulose produced the highest enzyme activity for mixed substrate of around 5.5 IFPU/ml in 6.5 days. However, growth of the fungus on cellulose alone attained a higher enzyme titer of 7 IFPU/ml in a time period of 6 days. Since the maximal productivity of the 30:30 combination was the highest (122 IFPU/l/hr) of all experiments and its total productivity reached maximum value of 55 IFPU/l/hr in less time than others (Figure 3), this ratio seems to be the optimal ratio of xylose to cellulose for enzyme production on mixed substrate in a batch system. The results of this work showed that increasing the total substrate (cellulose or a mixture of xylose and cellulose) concentration above 60 g/l does not improve high titer enzyme activity and productivity of enzyme production. This result is consistent with the results of Sternberg and Dorval (1979), Ghose and Sahai (1979) and Hendy et al (1972) for cellulose alone.

Fed-Batch Cultivation

In the second phase of the project the RUT C-30 mutant was grown in a fed-batch system with a start up and an intermittent addition mixture as shown in Table 2. Figure 5 shows an example of the growth pattern of RUT C-30 started on the mixture of 30:20 g/l xylose to cellulose, followed by intermittent addition of 5:15 g/l/day xylose to cellulose. Summary of the overall results for fed-batch system is shown in Figure 6. The advantages of fed-batch cultivation was to utilize high substrate levels with reduced production of cell mass compared to batch cultivation, which ensures adequate agitation and aeration. During the fed-batch cultivation the biomass builds up to certain level then stays constant after which the carbon source added is used for cell maintenance and enzyme production.

Figure 7, compares batch cultivation with 30:20 g/l xylose to cellulose, versus fed-batch cultivation with 30:20 g/l xylose to cellulose start up mixture and intermittent addition of 10:10 g/l/day xylose to cellulose. It can be seen from this figure that high titer activity was improved almost 57%, and the productivity gained 25%, as compared to the batch system. The results reported by other researchers have shown better improvement in high titer activity and productivity as compared to ours. This difference can be due to several reasons including method of enzyme measurement, agitation rate, antifoam addition, etc.

Total productivity, high titer enzyme activity and IFPU yield, defined as IFPU/gm carbon source used, for all fed-batch experiments are summarized in Table 4. Comparing the IFPU yield for experiments #1, 2, and 3, in which only cellulose was used as the intermittent feeding mixture, it can be seen that

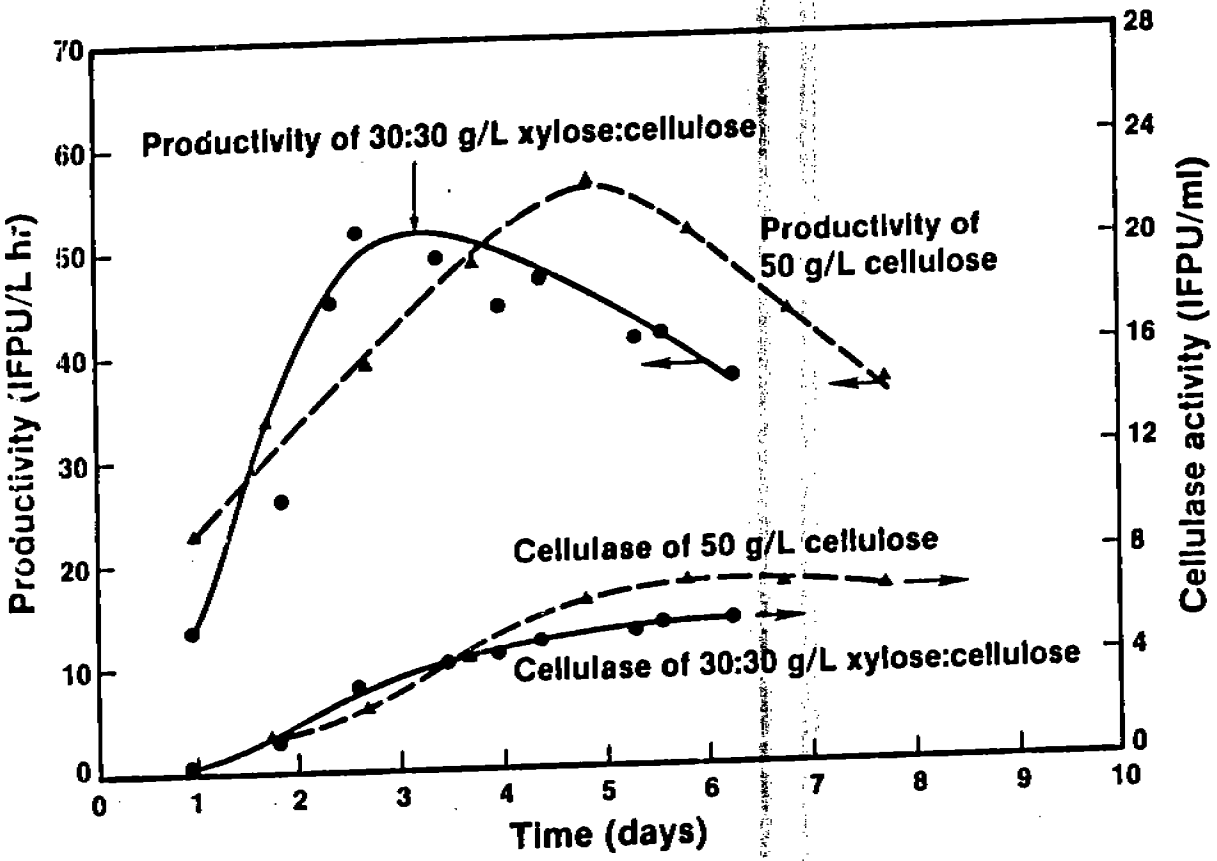



Figure 4: Productivity and Cellulase Activity as a Function of Time for T.reesei, RUT-C30 Grown on 50 g/l Cellulose and 30:30 g/l Xylose:Cellulose in Batch Culture at pH = 4.8, T = 28°C.

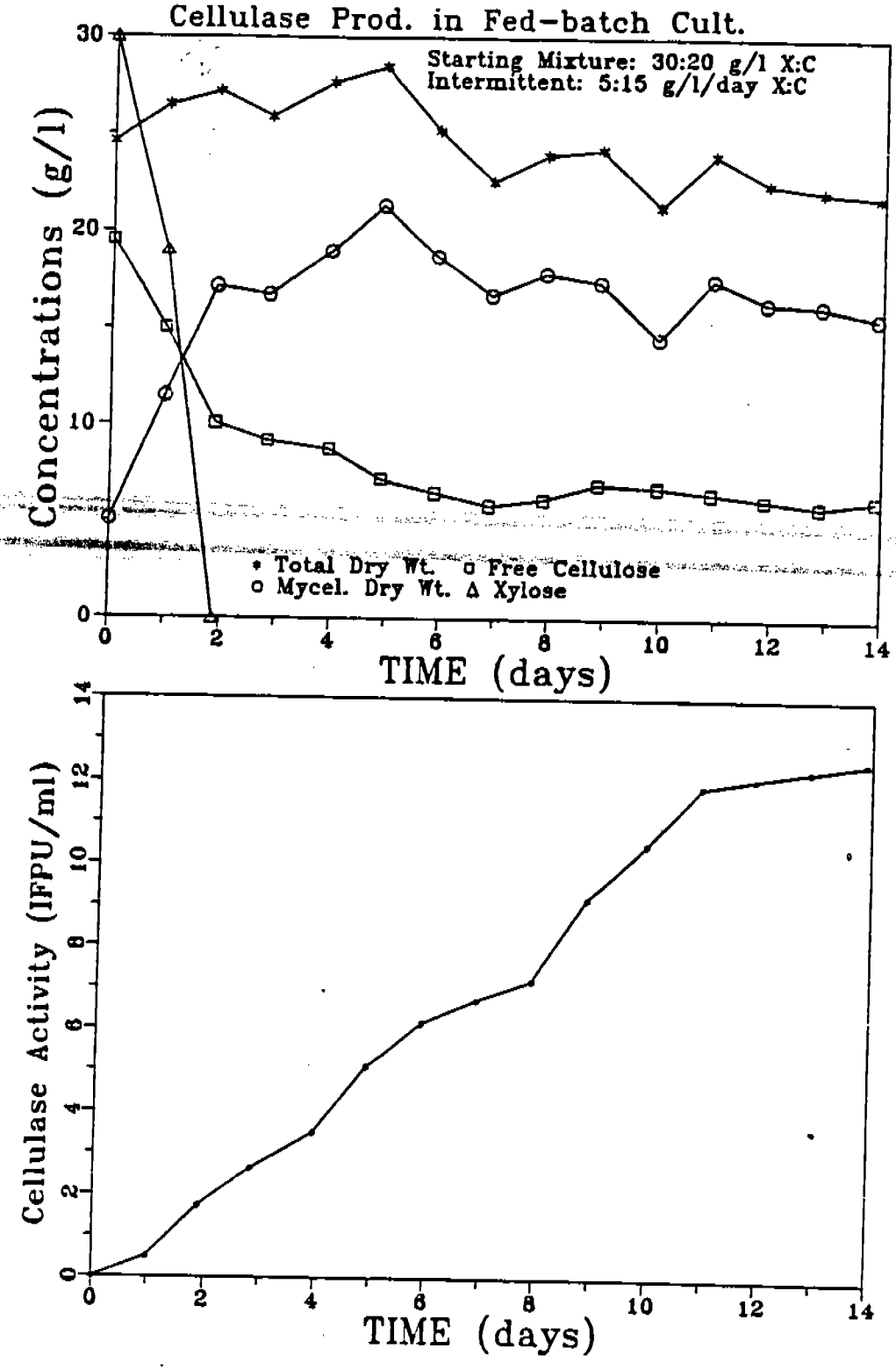



Figure 5: Growth and Enzyme Production Pattern of T. reesei RUT-C30 Grown on Mixture of Xylose and Cellulose in Fed-Batch Culture at pH = 4.8, T = 28°C.
Start up Mixture: 30:20 g/l Xylose:Cellulose
Intermittent Mixture: 5:15 g/l day Xylose:Cellulose

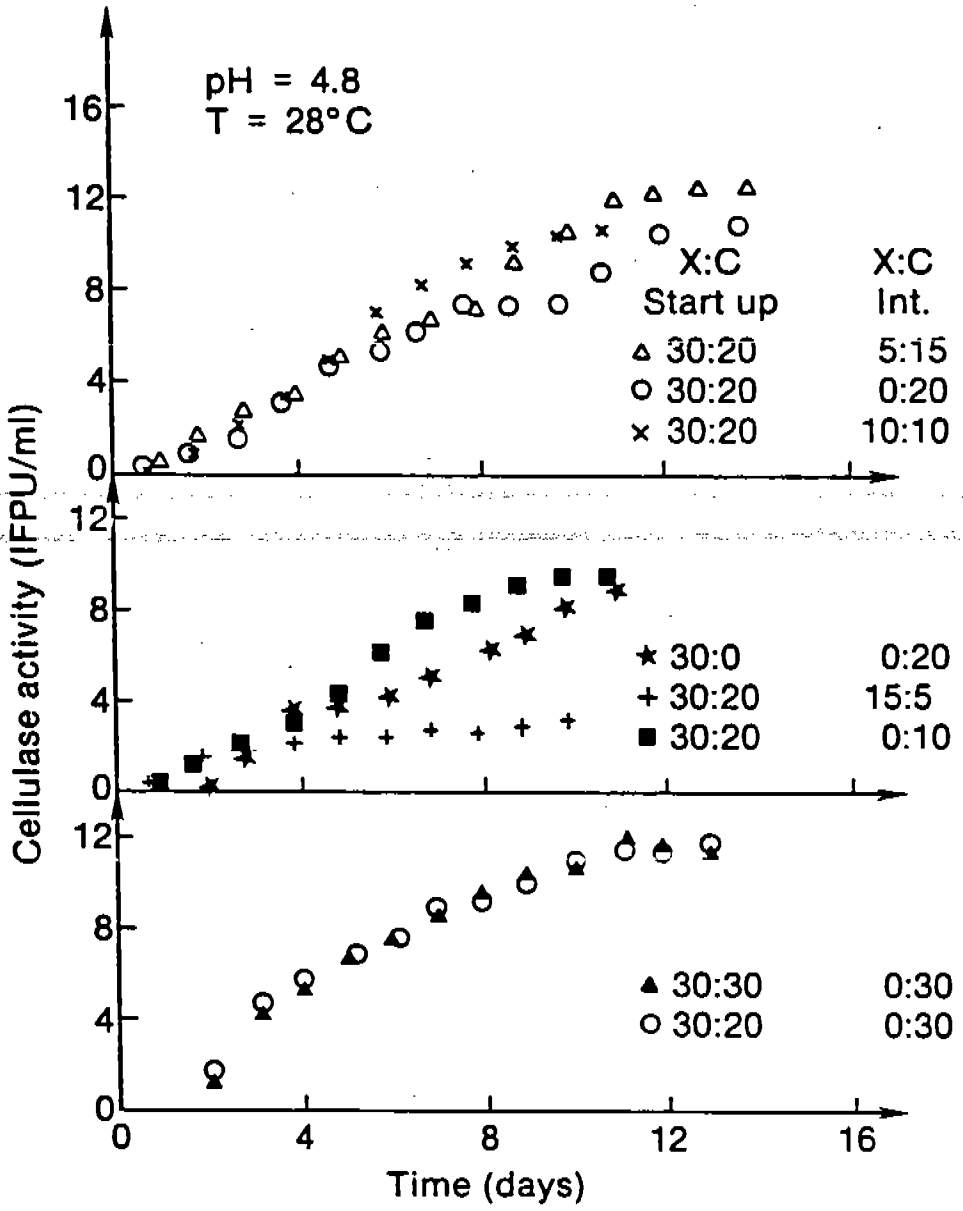
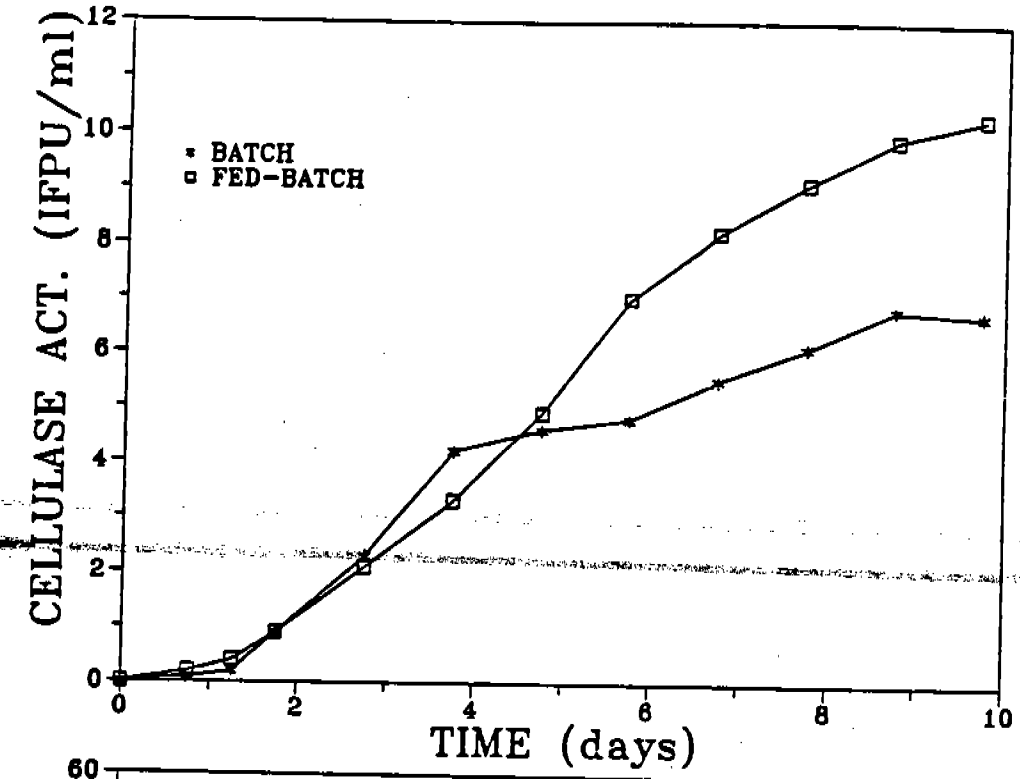



Figure 6: Cellulase Production as a Function of Time by T. reesei RUT-C30 Grown on Xylose/Cellulose Mixtures in Fed-Batch Culture at pH = 4.8, T= 28 C.



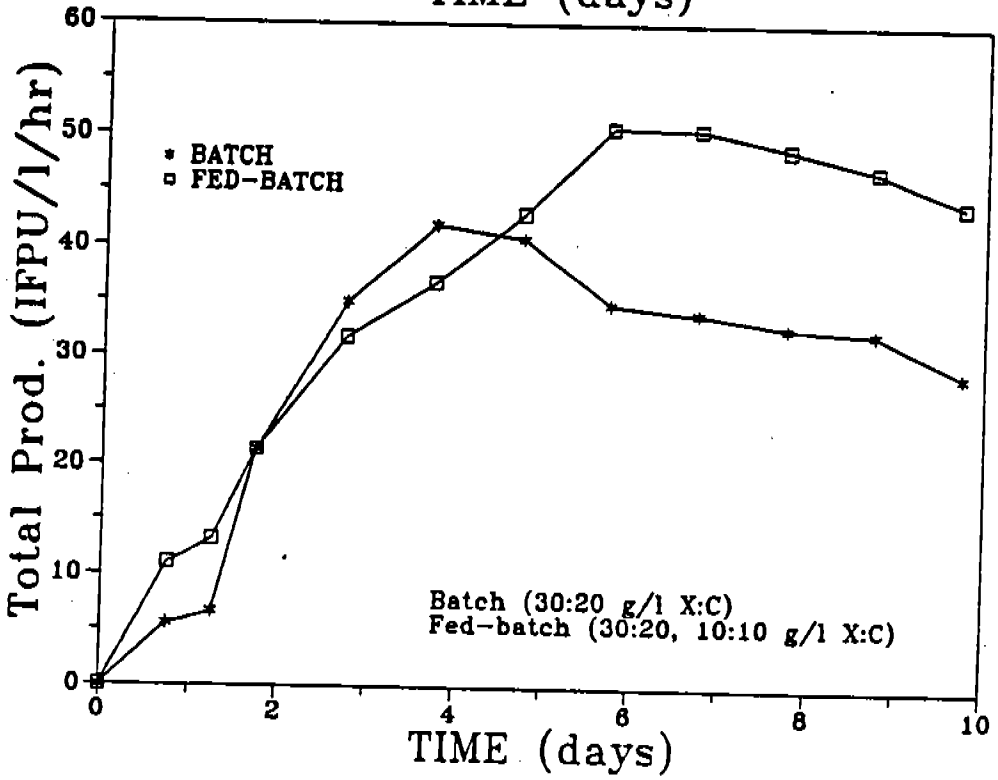




Figure 7: Comparison of Cellulase Activity and Productivity of Enzyme Production by T. reesei RUT-C30 in Batch and Fed-batch Culture, at pH = 4.8, T = 28°C.

TABLE 4: SUMMARY OF RESULTS ON THE HIGH TITER FILTER PAPER ACTIVITY, AND CORRESPONDENT PRODUCTIVITY OF RUT C-30 GROWN ON XYLOSE AND CELLULOSE IN A FED-BATCH SYSTEM

| Exp. # | Xylose:Cellulose Ratio (g/l) | | High Titer Activity IFPU/ml | Total Productivity IFPU/l/hr | Total C-Source Added gm/l | IFPU Yield IFPU/gm Total Carbon |
|---|---|---|---|---|---|---|
| | Start-up | Int. | | | | |
| 1 | 30:20 | 0:10 | 9.5 | 46.3 | 130 | 73.1 |
| 2 | 30:20 | 0:20 | 10.8 | 40.3 | 250 | 43.2 |
| 3 | 30:20 | 0:30 | 12.0 | 58.5 | 350 | 34.3 |
| 4 | 30:20 | 5:15 | 12.5 | 45.4 | 250 | 50.0 |
| 5 | 30:20 | 10:10 | 10.5 | 50.7 | 250 | 42.0 |
| 6 | 30:20 | 15:5 | 3.1 | 33.2 | 250 | 12.4 |
| 7 | 30:0 | 0:20 | 8.8 | 38.7 | 230 | 38.3 |
| 8 | 30:30 | 0:30 | 11.6 | 61.2 | 360 | 32.2 |

TABLE 5: COMPARISON OF IFPU YIELD* OF BATCH AND FED-BATCH CULTIVATION

| BATCH | | | FED-BATCH | | | |
|---|---|---|---|---|---|---|
| Xylose:Cellulose Ratios | Total C-Source Used (gm/l) | IFPU* Yield | Xylose:Cellulose Ratios | | Total C-Source Used (gm/l) | IFPU* Yield |
| | | | Start-up | Int. | | |
| 40:0 | 40 | 32.5 | 30:20 | 0:10 | 130 | 73.1 |
| 30:5 | 35 | 55.7 | 30:20 | 0:20 | 250 | 43.2 |
| 30:10 | 40 | 87.5 | 30:20 | 0:30 | 350 | 34.3 |
| 20:20 | 40 | 130.0 | 30:20 | 5:15 | 250 | 50.0 |
| 25:25 | 50 | 108.0 | 30:20 | 10:10 | 250 | 42.0 |
| 30:20 | 50 | 138.0 | 30:20 | 15:5 | 250 | 12.4 |
| 30:30 | 60 | 93.3 | 30:0 | 0:20 | 230 | 38.3 |
| 50:50 | 100 | 74.0 | 30:30 | 0:30 | 260 | 32.2 |
| 0:40 | 40 | 132.5 | | | | |
| 0:50 | 50 | 142.0 | | | | |
| 0:100 | 100 | 67.0 | | | | |

*IFPU/gm C-Source

0:10 g/l/day xylose to cellulose intermittent feeding mixture gave the best result, 73.1 IFPU/gm C-source. In another case, by comparing the IFPU yield of experiments #2, 4, 5, and 6 the effect of addition of xylose to feeding mixture can be determined. These results show that 5:15 feeding mixture gave a higher yield than 0:20 mixture. Also, it can be seen that as xylose concentration in the feeding mixture was increased to 10 and 15 g/l/day the yield decreased, which indicates that xylose addition suppresses cellulase enzyme production. In another case, comparing the result of experiment #2, and 5 shows that when 50% of cellulose was replaced with xylose, the high titer enzyme activity and IFPU yield remains the same, but the productivity increases by 20%. This result, along with the results in batch culture, shows that at least 50% of the cellulose can be replaced both in the start up and the intermittent feeding mixture without lowering the high titer activity or total productivity of the enzyme.

## DISCUSSION

The results show that the RUT C-30 mutant of T. reesei can be successfully grown on mixtures of xylose and cellulose. Xylose, which can be readily obtained from pretreatment of real lignocellulosic materials as a coproduct, supports the initial fungal growth. By supplementing the medium with a smaller amount of cellulose as an inducer, about the same productivity of enzyme can be obtained as when all of the substrate is cellulose.

In order to evaluate the economic advantage of this process, we can compare the result of experiment #4 (20:20 mixture of xylose to cellulose) with that of experiment #9 (40 g/l cellulose) of batch culture. It can be seen that by substituting 20 g/l of xylose for cellulose (50%), the maximal productivity has improved more than 100%, the total productivity has increased by 14%, the IFPU yield has remained the same (Table 5), and the lag phase of enzyme production has decreased. In another case, compare the result of experiment #9, (40 g/l cellulose) with that of experiment #3 (30:10 mixed substrate) in batch culture. In this case, we have substituted xylose for 75% of the cellulose. Total productivity has remained almost the same, the maximal productivity has improved by about 30%, and the lag phase for enzyme production has decreased substantially.

The results of fed-batch cultivation also confirms the economical advantages of this process. Comparing the result of experiment #2 with that of experiment #4 both with the same start up mixture of 30:20 g/l and intermittent feeding mixture of 0:20 and 5:15 g/l/day respectively, it can be seen that by substituting 25% of cellulose with xylose in the intermittent feeding mixture, the high titer activity has increased 16%, productivity has improved by 25%, and IFPU yield has gained 25% which shows the economical advantage of replacing some portion of cellulose with xylose in the intermittent feeding mixture. Also, as mentioned in result section, by replacing up to 50% of cellulose with xylose both in the start up and the intermittent feeding mixture, the high titer enzyme activity and productivity does not decrease as compared to cellulose alone.

It can be concluded that for enzyme production, replacing part of the cellulose with xylose improves maximal productivity in both batch and fed-

batch cultures, as compared to the same processes under similar conditions, and the same total productivity can be achieved in less time. The amount of cellulose saved by substituting xylose for it in enzyme production can be used for ethanol production. As a result, the substrate cost can be decreased substantially, making the process more economical.

Finally, in Table 5 we have compared the IFPU yield of the batch and the fed-batch system. This variable, which is an important one, has been ignored by many other researchers. It is useful for comparing the advantage of batch and fed-batch cultivation over each other. Table 5 shows that, even though the fed-batch system improves the high titer activity and the total productivity of enzyme production, the IFPU yield on the substrate consumed decreases, indicating that the IFPU yield is potentially being sacrificed for better productivity in the fed-batch system.

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